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Relaxin decreases renal interstitial fibrosis and slows progression of renal disease[1]

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Relaxin decreases renal interstitial fibrosis and slows progression of renal disease.

Background. Relaxin, a hormone of the insulin-growth factor family, promotes collagen remodeling. In rodent models of pulmonary and dermal fibrosis, relaxin reduced interstitial fibrosis. To study relaxin's effect in renal disease, we used the experimental bromoethylamine (BEA) model that leads to severe renal interstitial fibrosis, a decrease in glomerular filtration rate, and albuminuria at one month.

Methods. Rats were injected with BEA one week prior to implantation of an osmotic pump delivering relaxin (2 μg/hour) or vehicle continuously for 28 days.

Results. BEA caused a significant decrease in creatinine clearance, which was partially prevented by relaxin. In the relaxin-treated BEA rats, serum creatinine was normal, and albumin excretion was slightly decreased. By morphometric measurement, relaxin administration was associated with a significant decrease in interstitial fibrosis at the corticomedullary junction. This was accompanied by a decrease in the number of ED-1 positive cells (an index of macrophage infiltration) and in the intensity of immunohistochemical staining for transforming growth factor-β. This antifibrotic effect of relaxin did not appear to be mediated by systemic hemodynamic changes since the mean arterial pressure was not significantly different among the groups.

Conclusions. Relaxin may have a useful application in decreasing interstitial fibrosis and thereby slowing the progression of renal disease.

Efforts to prevent the progression of renal diseases have relied largely on dietary protein restriction and the use of antihypertensive drugs, especially angiotensin-converting enzyme (ACE) inhibitors. In previous studies, we have shown that the ACE inhibitor enalapril reduced interstitial fibrosis, decreased the production of transforming growth factor-β (TGF-β), and increased gelatinase activity in the bromoethylamine (BEA) model of renal fibrosis [1, 2]. In this model, an initial papillary necrosis is followed by extensive interstitial fibrosis and renal insufficiency [3].

Relaxin, a hormone of the insulin growth factor family, has a broad range of biologic activities, including induction of collagen remodeling mediated by effects on collagen and collagenase synthesis [4, 5]. The purpose of this study was to determine the effect of a continuous infusion of relaxin on the progression of BEA-induced renal papillary necrosis and interstitial fibrosis.

METHODS

Experimental design

Male Sprague-Dawley rats (Harlan), weighing 250 g at the beginning of the study, were housed individually on a standard 12:12 light-dark cycle with free access to standard rat chow (PMI #5012; PMI Feeds, Inc., St. Louis, MO, USA) and drinking fluid. The animals were conditioned to handling and tail-cuff blood pressure measurement (IITC Life Science, Woodland Hills, CA, USA) for three weeks prior to the experimental period. All animals had a stable blood pressure at that time. The experiments were conducted in accord with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the institutional review board.

Groups of conditioned rats were injected intraperitoneally with BEA (200 mg/kg body weight) or vehicle (0.9% sterile saline). Renal balance studies were done at one-week post-BEA to insure that all animals had papillary necrosis, as evidenced by increased urinary output and decreased urine specific gravity. Animals were then randomly assigned to treatment groups. Osmotic

[1] **See Editorial by Becker and Hewitson, p. 1184.**

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minipumps (Alzet model 2 ML4; Alza Corp., Palo Alto, CA, USA) filled with either relaxin [Recombinant human relaxin, ConXn™; 2 μg/h; Connetics Corp., Palo Alto, CA, USA (Rlx)] or vehicle (20 mmol/L sodium acetate, pH 5.0) were implanted subcutaneously between the scapulae. Blood pressures were measured twice weekly for the next 26 days. At that time, a second minipump containing ^{14}C inulin was placed in the peritoneal cavity. Animals were allowed to recover for 24 hours, and two 24-hour urine collections with midpoint blood collections were obtained for measuring glomerular filtration rate (GFR) in conscious animals [6, 7]. At day 28, another 24-hour urine collection was obtained for creatinine clearance. Following this, animals were anesthetized with sodium pentobarbital (50 mg/kg), and blood samples were taken from the descending aorta and renal vein. The kidneys were perfused retrogradely with cold saline. One kidney was removed for collagenase studies, and the other was perfused in situ with 50 mL of HistoChoice (Amresco Inc., Solon, OH, USA) and processed for routine histology.

Biochemical studies

Urine and plasma creatinine and inulin were measured by standard methods. Urine albumin was measured by radial immunodiffusion on plates obtained from The Binding Site (Birmingham, UK).

Relaxin enzyme-linked immunosorbent assay (ELISA)

Serum relaxin levels were quantitated by a sandwich immunoassay [5]. Briefly, wells of a microtiter plate (Maxisorp Immunomodules; Nunc, Naperville, IL, USA) were coated overnight with affinity-purified goat anti-human recombinant relaxin polyclonal antibody. After an overnight incubation, wells were washed, and 100 μL of diluted affinity-purified rabbit anti-human recombinant relaxin polyclonal antibody conjugated to peroxidase were added to each well. Following an appropriate incubation period and color development with tetramethylbenzidene, the reaction was stopped by acidification. Absorbencies at 450/630 nm were measured, and concentrations of individual sera were determined by entering data into a four-parameter logistic curve-fitting program. A standard curve of recombinant human relaxin ranging from approximately 12 to 750 pg/mL was run on each assay plate. The assay has been validated for use with rat serum (that is, recovery, linearity of dilution, lower limit of detection, intra-assay, and interassay precision) and has a sensitivity of approximately 15 pg/mL. Sera were run at no less than a 1:20 dilution; therefore, the sensitivity in serum is 300 pg/mL.

Immunohistochemistry

Macrophages were identified with a mouse monoclonal antibody to ED-1 (Harlan Bioproducts, Indianapolis, IN, USA). The secondary antibody was conjugated to alkaline phosphatase and visualized with Fast Red Naphthol (Sigma, St. Louis, MO, USA). Positively stained cells were counted in 20 adjacent ×40 fields at the corticomedullary junction and in 50 glomeruli.

The primary TGF-β antibody was a rabbit polyclonal obtained from Promega Corp. (Madison, WI, USA). As previously discussed in this article, an alkaline phosphatase-Fast Red Naphthol detection system was used. Staining intensity was quantitated on digitized images using Sigma Scan (SPSS, Chicago, IL, USA). In each section, intensity measurements were made of the tubules bordering the fibrosis, normal tubules, and the background. A ratio of staining intensities minus background was obtained for each section. This ratio was used for comparisons among groups.

Gelatinase assay

Gelatinase activity was quantitated by a fluorescence assay with gelatin FITC as the substrate as previously described [2, 8]. Tissue extracts were adjusted to 0.5 mg/mL protein for this assay. Fluorescence was measured at excitation 491 and emission 519. The amount of gelatin degraded was determined by comparing the samples to a standard prepared by maximally digesting 1 to 20 μg/mL of FITC gelatin with clostridial collagenase. Gelatinase activity as measured by this assay yields the net available gelatinolytic activity that is free of tissue inhibitors of metalloproteinase (TIMP) inhibition.

Morphometric analysis

Interstitial fibrosis was evaluated at the corticomedullary junction on Masson's trichrome-stained coronal sections. We defined this area as that portion of the cortex inferior to the juxtamedullary glomeruli and consisting of the outer stripe and the superior portion of the inner stripe of the outer medulla. Care was taken not to include the inner medulla in these measurements. Contiguous fields encompassing the total C-M junction were photographed at ×4 and were digitized. The area of fibrosis, including the interstitial cell infiltrate and atrophic tubules, was measured. Papillary necrosis was measured on periodic acid-Schiff–stained digitized sections. These images included the tip and approximately three fourths of the papilla. This area was chosen because only in the animals receiving BEA alone did the lesion extend beyond this point. Measurements were made using Sigma Scan (SPSS) and were expressed as a percentage of the total area.

Statistics

Except as indicated, data were expressed as mean ± SE. Statistical analyses [analysis of variance (ANOVA), regression analysis, and *t* test] were performed where appropriate using SigmaStat (SPSS).

Table 1. Urine specific gravity and output

Treatment	Specific gravity before relaxin	Specific gravity after 28 days of relaxin	Urine volume *mL/hr* after 28 days of relaxin
None $N = 8$	1.026 ± 0.005	1.032 ± 0.003	1.11 ± 0.14
Relaxin $N = 6$	1.031 ± 0.005	1.037 ± 0.008	0.87 ± 0.26
BEA $N = 11$	1.017 ± 0.001[a]	1.017 ± 0.002[a]	2.18 ± 0.23[a]
BEA + relaxin $N = 15$	1.018 ± 0.001[a]	1.019 ± 0.002[a]	1.86 ± 0.12[a]

[a]Significantly different from control at $P \leq 0.005$

Table 2. Mean arterial pressures (mm Hg) at representative time points

Treatment	1 week after BEA	1 week after relaxin	Final
Control	116 ± 2	114 ± 6	117 ± 1
Relaxin	119 ± 3	116 ± 9	123 ± 3
BEA	109 ± 6	98 ± 8	114 ± 4
BEA + relaxin	118 ± 4	113 ± 4	110 ± 7

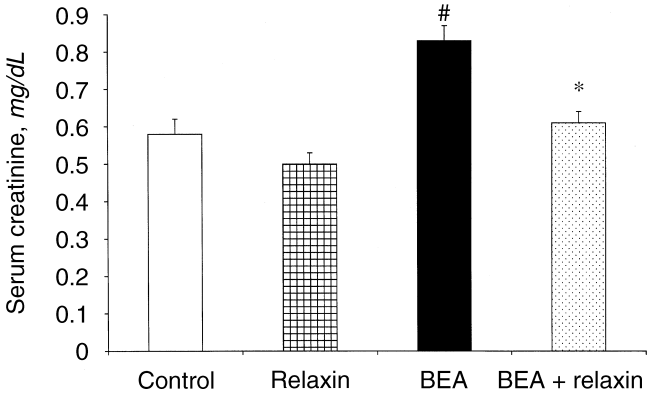


Fig. 1. Comparison of serum creatinine at five weeks. Bromoethylamine (BEA) + relaxin treatment: BEA one week before, then relaxin treatment for four weeks. *$P < 0.05$ compared with BEA alone; #$P < 0.05$ compared with the control.

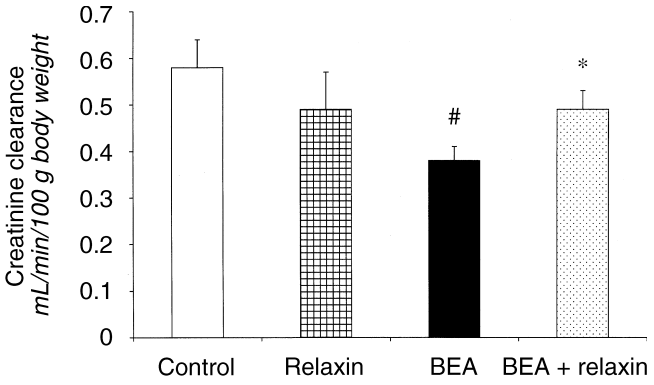


Fig. 2. Comparison of glomerular filtration rate, shown here as creatinine clearance. BEA + relaxin treatment: BEA one week before, then relaxin treatment for four weeks. *$P < 0.05$ compared with BEA alone; #$P < 0.05$ compared with the control.

RESULTS

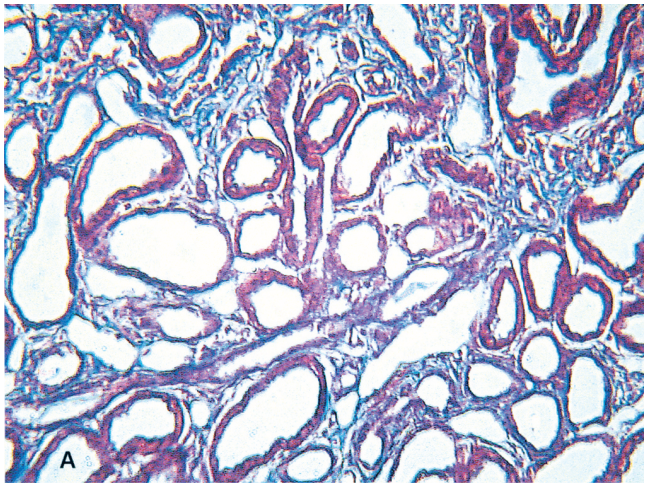
Effect of treatment with relaxin on functional parameters

One month after BEA injection, there was complete necrosis of the papilla with interstitial fibrosis, a mononuclear cell infiltrate, and tubular atrophy. As seen in Table 1, animals receiving BEA, with or without relaxin treatment, had a significant concentrating defect, indicating that papillary necrosis had taken place. Relaxin alone did not affect the urine-specific gravity or induce a diuresis at any time during the experimental period. Serum relaxin levels in the control and untreated BEA animals were below the detectable limit of the assay. The levels in animals receiving relaxin alone or in the BEA + relaxin group were comparable (Rel 152 ± 87 vs. BEA + Rel 129 ± 69 ng/mL, P = NS). Representative time points of tail-cuff blood pressures are shown in Table 2. ANOVA of the mean arterial pressure (MAP) revealed no significant differences within or between any groups over the time course of the study.

As expected, in animals receiving only BEA, the serum creatinine rose to 0.83 ± 0.04 mg/dL, a 30% increase over control. In the animals receiving relaxin, serum creatinine levels were indistinguishable from those of the controls (controls 0.58 ± 0.04 vs. BEA + Rel 0.61 ± 0.03 mg/dL; Fig. 1). In the BEA group, the GFR, as measured by creatinine clearance, decreased by approximately 33% as compared with controls (controls 0.58 ± 0.06 vs. BEA 0.34 ± 0.03 mL/min/100 g body weight, $P = 0.004$). The results of the inulin clearances were similar (controls 0.65 ± 0.13 vs. BEA 0.38 ± 0.02 mL/min/100 g body weight). Relaxin reversed this trend and restored creatinine clearance to approximately 85% of the control level (BEA 0.38 ± 0.03 vs. BEA + Rel 0.49 ± 0.04 mL/min/100 g body weight, $P = 0.044$; Fig. 2). There was also a decrease in albumin excretion in the treated animals (BEA 17.9 ± 5.5 vs. BEA + Rel 11.9 ± 2.8 mg/24 hours), but the difference did not achieve statistical significance.

Effect of relaxin treatment on interstitial fibrosis and its mediators

The kidneys of rats that received BEA showed complete papillary necrosis by one month. At this time, the normal parenchyma proximal to the papillary stump was replaced by fibrous tissue. In the relaxin-treated BEA animals, there was some degree of papillary necrosis, but this was less than that seen in animals receiving only



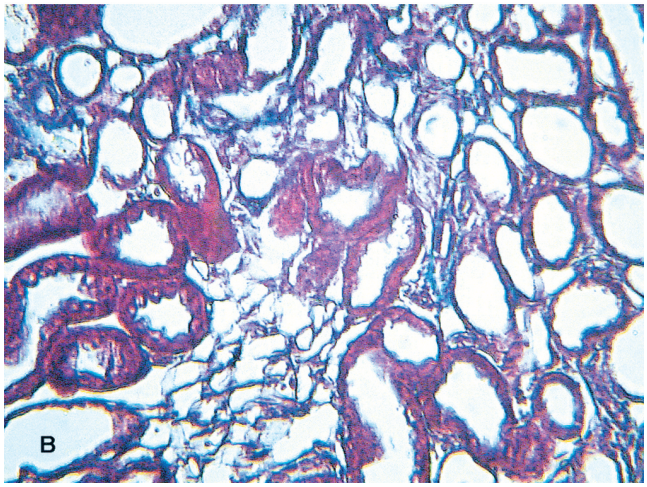


Fig. 3. Representative histology of the corticomedullary area at five weeks. (*A*) BEA alone. (*B*) BEA + relaxin. Masson's trichrome stain, ×10 at microscope.

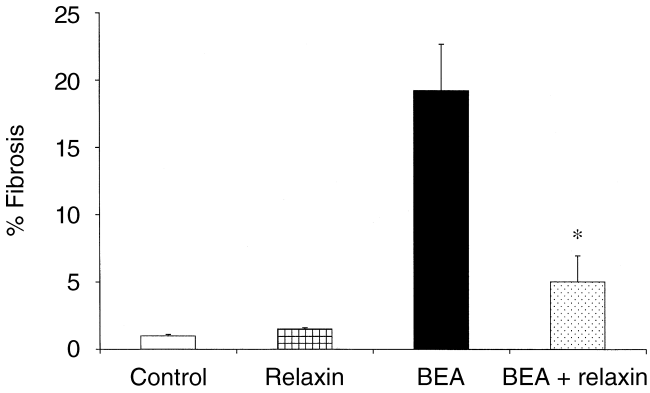


Fig. 4. Percentage of the corticomedullary area that showed fibrosis. *$P < 0.002$ as compared with BEA alone.

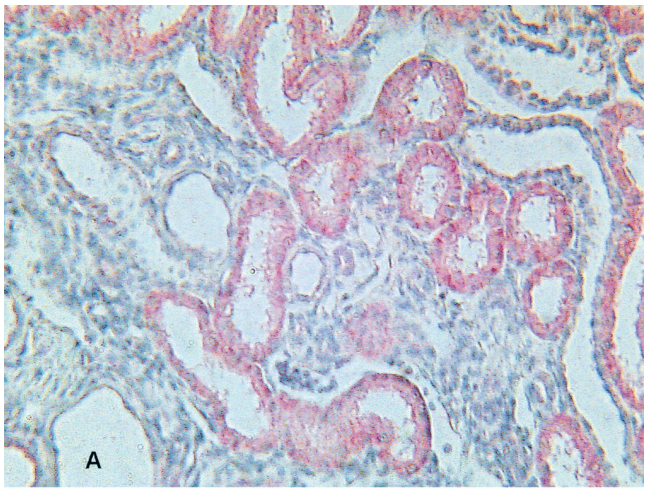
Table 3. Number of ED-1 positive cells in the corticomedullary junction (C-MJ) and glomeruli after one month of treatment

Treatment	*N*	C-MJ cells/mm^2	Cells/50 glomeruli
None	5	355 ± 80	4 ± 1
BEA	6	2336 ± 616[a]	8 ± 1
Relaxin	6	704 ± 120[a,b]	5 ± 2
BEA + relaxin	12	1043 ± 236[a,b]	8 ± 1

[a]Significantly different from Control at $P \leq 0.05$
[b]Significantly different from BEA at $P \leq 0.05$

BEA (BEA 100% in all animals versus 3 to 21% in the relaxin-treated rats).

At the corticomedullary junction in the BEA-treated rats (Fig. 3A), there was a marked interstitial fibrosis characterized by collagen deposition, a mononuclear cell infiltrate, tubular dilation, and tubular atrophy. In the cortex, many of the glomeruli showed thickening of Bowman's capsule. In a comparable section of the corti-



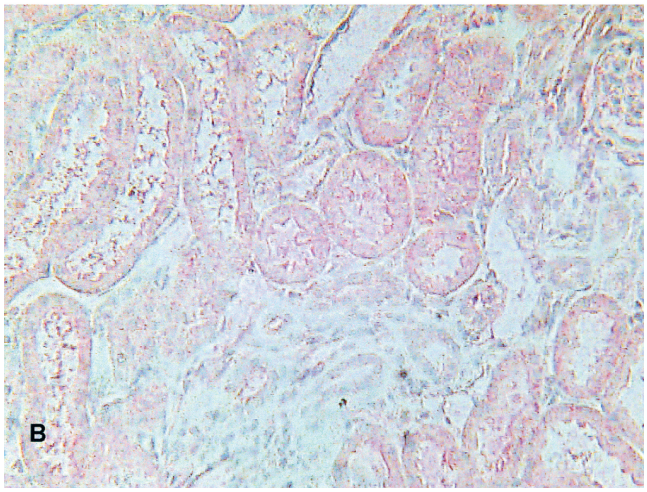


Fig. 5. Immunohistochemical staining for transforming growth factor-β (TGF-β). (*A*) BEA alone. There is an increase in the staining intensity in the tubules adjacent to the fibrosis. (*B*) BEA + Relaxin. There is an apparent decrease in staining intensity in the tubules. Fast red naphthol antibody visualization counterstained with hematoxylin (×10 at microscope).

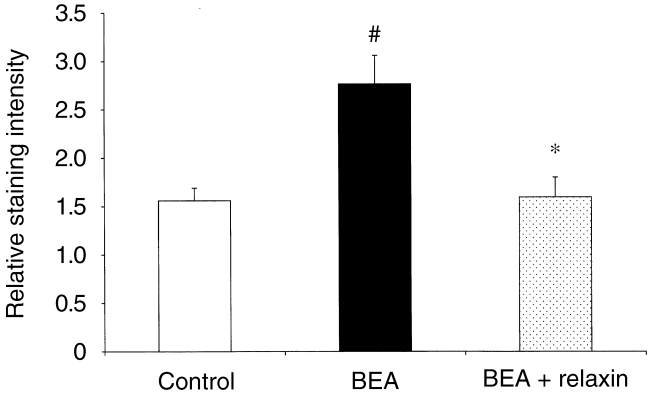


Fig. 6. Comparison of TGF-β staining intensities. Control 1.56 ± 0.13, BEA 2.77 ± 0.29, BEA + relaxin 1.60 ± 0.21. BEA + relaxin treatment: BEA one week before, then relaxin treatment for four weeks. *$P <$ 0.05 compared with BEA alone; #$P < 0.05$ compared with the control.

comedullary junction from a relaxin-treated BEA rat, the majority of the tubules were preserved, yet there were still some localized areas of increased collagen deposition and cellular infiltrate (Fig. 3B). Using morphometric techniques, we quantitated the percentage of the area at the corticomedullary junction composed of fibrous tissue. In the BEA animals, fibrosis accounted for approximately 20% of the corticomedullary junction (19.2 ± 3.4%, $N = 11$), whereas in the relaxin-treated animals, the fibrotic area comprised only 5% (5.0 ± 1.9%, $N = 11$, $P < 0.002$; Fig. 4). This is the equivalent of a 75% decrease in the extent of fibrosis.

There were no significant differences in the number of ED-1–positive cells in the glomeruli of any group. At the corticomedullary junction, BEA caused a sixfold increase in macrophage infiltration, indicative of a sustained chronic inflammation. In the relaxin-treated BEA animals, there was a decrease of over 50% in the number of these cells compared with BEA alone (Table 3).

There was a uniform basal level of TGF-β staining in the tubules in the control rat. The staining intensity was markedly increased in the tubules in BEA animals. Tubules adjacent to areas of fibrosis showed intensified but nonuniform staining for TGF-β (Fig. 5A), while the uninvolved areas of the kidney showed no increase in tubular staining. In the animals treated with relaxin, there was a decrease in tubular TGF-β staining, in association with an overall decrease in fibrosis (Fig. 5B). The heterogeneous staining pattern of TGF-β seen in Figure 5 would have made total kidney TGF-β levels difficult to interpret. For this reason, we measured the TGF-β staining intensities in the tubules next to the fibrosis and compared them with the normal tubules in the same section. These results are summarized in Figure 6. BEA-treated animals had a 77% increase in TGF-β staining as compared with the controls. In BEA animals receiving relaxin, the intensity of TGF-β staining was not different than the controls and was significantly lower than that of BEA-treated animals.

Relaxin alone did not alter the gelatinase activity levels compared with the controls (cortex 105 ± 4 vs. 103 ± 4; medulla 102 ± 12 vs. 97 ± 10 μg gelatin degraded/h/mg tissue protein, control vs. relaxin, respectively). As we have previously reported, BEA-induced fibrosis was accompanied by a significant decrease in gelatinase activity in both cortex and medulla. There were no changes in the animals treated with relaxin (cortex 64 ± 11 vs. 59 ± 4; medulla 58 ± 14 vs. 38 ± 4 μg gelatin degraded/h/mg tissue protein, BEA vs. BEA + relaxin, respectively).

DISCUSSION

Relaxin is a heterodimeric hormone of 6000 Daltons that belongs to the insulin growth factor family. It is found most abundantly in pregnancy but is also present in nonpregnant women and men. Relaxin has a broad range of biologic activities, including induction of collagen remodeling in fibrotic tissue, softening of the cervix at parturition, inhibition of uterine contractions, and growth of the mammary gland [9]. In human dermal fibroblasts, relaxin modulates collagen deposition by decreasing collagen synthesis and increasing its degradation [4]. Unemori et al have studied the effects of relaxin in human lung fibroblasts in vitro [5]. They found that relaxin inhibited TGF-β–induced procollagen and fibronectin overexpression in a dose-dependent fashion and was associated with increased collagenase activity. Their study also examined the effect of a continuous infusion of relaxin on bleomycin-induced pulmonary fibrosis in mice. The striking increase in the hydroxyproline content of the lung usually seen in that model was dramatically reduced by relaxin, and the histologic degree of pulmonary fibrosis was markedly attenuated. In clinical trials in patients with scleroderma, relaxin has been found to be safe and well tolerated [10], raising the possibility that relaxin might be clinically useful in diminishing collagen accumulation and fibrosis in human fibrotic diseases.

The BEA model of papillary necrosis is a convenient, reproducible method to study renal interstitial fibrosis. A single dose induces papillary necrosis that is histologically evident at seven days. At this time, there is a loss of the epithelium of the papillary tip, dissolution of the tubular structures within the papilla, and a mild interstitial fibrosis. In our current study, relaxin infusion was begun one week after the induction of papillary necrosis by BEA in order to elucidate whether relaxin would be able to inhibit the fibrotic process initiated by this compound.

Our results clearly show that in animals in which papil-

lary necrosis had been well established, relaxin administration prevented the progression of the necrosis and had a significant impact on preserving renal function, as evidenced by the restoration of creatinine clearance to 85% of control levels. This was comparable to the degree of improvement we have seen in animals treated with ACE inhibitors [1].

In parallel with relaxin's ability to preserve renal function, it also preserved renal structure. The relaxin-treated animals demonstrated a nearly normal renal architecture in contrast to the extensive fibrosis and tubular dilation caused by BEA alone. Relaxin administration was associated with a 75% decrease in the deposition of collagen in the corticomedullary junction. In association with the decreased fibrosis, tubular structure was also largely maintained. This is the first study to show a decrease in fibrosis in the kidneys with relaxin treatment. Indeed, the improvement in the degree of fibrosis was quite remarkable. One cannot help but hypothesize that the amelioration of fibrosis by relaxin was key to maintaining renal function despite the administration of BEA, a potent initiator of renal fibrosis.

The mechanism whereby relaxin exerted its protective effect was studied in detail. Blood pressure control plays an important role in the progression of many renal diseases. In our study, relaxin did not alter systemic blood pressure in control or experimental animals, suggesting that blood pressure was not a significant factor. Given that hypertension is not a prominent feature of this model, this was expected. Our findings are in agreement with those of Danielson, Sherwood, and Conrad, who also did not show a change in MAP with short-term relaxin infusion [11]. However, they did show an increase in GFR, which they attributed to renal nitric oxide production. We do not believe that the improvement in GFR seen in the relaxin-treated BEA animals was simply a hemodynamic effect, because normal animals infused with relaxin did not show an increase in GFR. Furthermore, the improvement in GFR was associated with a striking decrease in the extent of fibrosis, strongly suggesting a relationship of cause and effect between the histologic improvement and the increase in GFR.

The kidneys of BEA-treated animals had a tenfold increase in ED-1–positive cells, a cell type frequently associated with interstitial fibrosis and matrix synthesis. This increase was attenuated in the relaxin-treated BEA animals. The finding of a twofold increase in this cell type in those animals receiving only relaxin was unexpected, and we have no explanation for this finding. Although investigators have been unable to demonstrate relaxin receptors on resident macrophages in rodent lung or peritoneum [12], this does not rule out the possibility of their presence in the resident kidney macrophages.

Matrix synthesis and degradation in many tissues is regulated by TGF-β, and such regulation may be altered in various diseases [13]. Studies have now shown that up-regulation of TGF-β is associated with an increase in matrix accumulation in several fibrotic renal diseases [14, 15]. In our earlier studies, we demonstrated an increase in TGF-β expression in the BEA model [1, 2]. In the present study, four weeks of relaxin administration was accompanied by a decrease to control levels in the tissue expression of TGF-β, suggesting that relaxin may protect against renal scarring. Unemori et al have previously shown in vitro that relaxin inhibits TGF-β's fibrotic actions in a dose-dependent manner [5]. Our finding of decreased TGF-β staining intensity in the treated animals extends that observation to an in vivo model.

One mechanism by which relaxin is thought to prevent fibrosis is by increasing collagenase activity, as shown in experiments using human lung fibroblasts in vitro [9]. In an earlier study, we have shown that BEA administration is associated with an early (first day) decrease in medullary gelatinase activity, which remained suppressed for the 30 days of the study [2]. In the present study, we confirmed that BEA-induced interstitial fibrosis was associated with a decrease in gelatinase activity, which was not altered by relaxin. These observations were made at 30 days and therefore do not preclude an earlier change in gelatinase activity or changes in interstitial collagenase activity that may be important in preventing the onset of fibrosis.

In conclusion, in this model of chronic renal interstitial fibrosis, relaxin administration resulted in an improvement in GFR, a decrease in the number of ED-1 positive cells, a decrease in immunohistochemical staining of TGF-β, and a decrease in the amount of fibrosis in the kidney. The ability of relaxin to prevent the progression of fibrosis, as measured both functionally and histologically, makes it tempting to suggest that relaxin has promise as a therapeutic agent for the treatment of a multitude of renal diseases having interstitial fibrosis as a part of their pathologic picture.

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